bio-protocol Preprint

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Isolation and methylation profiling of cfDNA

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An abbreviated version of this protocol was published in Science Translational Medicine in Dec 2019

Circulating tumor DNA methylation profiles enable early diagnosis prognosis prediction and screening for colorectal cancer

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Detailed protocol

The Vacuum-driven extraction of cfDNA from Human Serum or Plasma

In this program, we used the optimized protocol of HiPure Circulating DNA Midi Kit(Magen, China) to extract cfDNA from human serum or plasma.

Procedure:

1. Pipette 2.0 mL serum/plasma into a 15 mL centrifuge tube. If the sample is less than 2 mL , please adjust the volume of regents(Proteinase K, Buffer ACL and ACB) in proportion in step 2-4.
2. Add 0.2mL Proteinase K, vortex for 5s to mix well.
3. Add 1.6mL Buffer ACL into the sample tube, invert the tubes 20 times to mix well. Incubate at 60°C water bathing for 30 minutes.
4. Add 3.6mL Buffer ACB(Isopropanol added) into the sample tube, gently invert the tubes 20 times to mix solution thoroughly. Keep the sample on ice for 5 minutes.
5. Insert the Hipure CFDNA Mini Column into the vacuum manifold and insert the Extender Tube into the Column. Slowly pour all the sample solution from step 4 into the Extender Tube.
6. Open the vacuum (0.2 bar) and allow all the sample solution flow through the column.
7. Close the vacuum and release the pressure to zero. Add 0.6mL Buffer DCW1 (Ethanol added) into the column. Open the vacuum (0.4 bar) and allow all the buffer flow through the column.
8. Close the vacuum and release the pressure to zero. Add 0.6mL Buffer DCW2 (Ethanol added) into the column. Open the vacuum (0.4 bar) and allow all the buffer flow through the column.
9. Close the vacuum and release the pressure to zero. Add 0.6mL 100% Ethanol into the column. Open the vacuum (0.4 bar) and allow all the buffer flow through the column. Close the vacuum.
10. Close the column cap and place the Column in the Collection Tube, and centrifuge at 13,000 rcf for 3 minutes. Discard the Collection Tube.
11. Place the Column in a new 1.5 mL microcentrifuge tube. Open the Column cap and air-dry for 10 minutes.
12. Apply 50 µl Nuclease Free Water (60°C) to the center of the membrane. Close the cap and incubate at RT for 5 minutes. Centrifuge at 13,000 rcf for 1 minute.
13. Discard the column, store the DNA at 2-8°C for immediate use or -20°C for long-term storage.

ddPCR™ Protocol

I. Required Materials

(Bio-Rad) Droplet Generation Oil for Probes (186-3005)
(BioRad) DG8 Gaskets for QX200 Droplet Generator (186-3009)
(Bio-Rad) DG8 Cartridges fro Droplet Generator (186-4008)
(Bio-Rad) ddPCR Supermix for Probes (No dUTP) (186-3023/3024 depending on size) (Bio-Rad) Pierceable Foil Heat Seal (181-4040)
(Fisher) Eppendorf twin.tec 96-well plates, semi-skirted (951020346)

II. PCR Set-Up

1. Dilute bis-DNA samples to 5~20ng.
2. Prepare the reaction master mix with water, ddPCR™ Supermix for Probes, primes, Taqman FAM/HEX probes.

		Reaction Master Mix for N Samples
Per Reaction		
DNA	X	
2x Supermix	10 uL	x N
10uM FAM probe	0.2 uL	
10uM HEX probe	0.2 uL	

10uM	Primer F/R	0.8 ul
H_2O		8.8-X
Total = 20 uL		

3. Since Droplet Generation must be done in an 8-chamber format, if the total number of samples to be analyzed is not a multiple of eight, the remaining empty tubes of the PCR strip must be filled. Add 12 uL ddPCR™ Buffer Control Kit (2x) and 12 uL water to each empty tube.
4. Spin the PCR strip/plate down.
5. See Ⅲ. Droplet Generation Section.
6. Run foil-sealed PCR plate on thermocycler with the following program:
   98°C 10 min;(94°C 30 sec, 53°C 1 min) x 40 cycles;98°C 10 min;12°C forever. All steps at 2ºC/s ramp rate.
7. Load plate into QX100™ Droplet Reader and analyze.

Ⅲ. Droplet Generation

1. Set aside an Eppendorf twin.tec 96-well PCR plate and cover with a piece of Pierceable Foil Heat Seal. Make sure the red line on the foil faces upward.
2. Load a DG80™ Cartridge into the Cartridge Holder and close the holder.
3. Using a multi-channel pipettor set to 20 uL, gently pipette the reaction mixture in the 8-tube PCR strip up and down to mix. Take care not to create any bubbles. Transfer 20 uL reaction mixture from the 8-tube PCR strip/96 well plate into the middle row of chambers on the Cartridge designated for “Sample”. Remove any bubbles in the chambers with a P20 micropipettor. The instance of bubbles drastically reduces droplet count in the generation step. Take note of orientation of the cartridge with respect to sample order to ensure correct loading of the final 96-well PCR plate.
4. Add 70 uL Droplet Generator Oil to the bottom row of the cartridge designed for “Oil.” Do not load oil before samples as this will reduce droplet number.
5. Fit rubber DG80™ Gasket onto Cartridge by catching two holes on either end of the Gasket with coordinated hooks on the edges of the Cartridge Holder.
6. Gently place Cartridge holder into the QX1000™ Droplet Generator. Droplet generation should take about 1 minute.
7. Remove the Cartridge Holder from the machine and discard Gasket. Droplets are held in the top row of the Cartridge and slightly opaque. Use a multi-channel pipettor set to 45uL to transfer droplets into a column of the Eppendorf twin.tec 96-well PCR plate. Do not press the pipettor tightly to the bottom of the cartridge or pipette to vigorously as this will shear the droplets.
8. Cover the PCR plate with the foil sheet immediately after to reduce the risk of contamination.
9. Repeat steps 2-8 to generate droplets for all your samples.
10. Load the 96-well plate and foil seal into the PX1™ PCR Plate Sealer set to 180°C and seal plate.

Related files

bisulfite conversion protocol.pdf

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Luo, H. and Xu, R. (2020). Isolation and methylation profiling of cfDNA. Bio-protocol Preprint. bio-protocol.org/prep245.
2. Luo, H., Zhao, Q., Wei, W., Zheng, L., Yi, S., Li, G., Wang, W., Sheng, H., Pu, H., Mo, H., Zuo, Z., Liu, Z., Li, C., Xie, C., Zeng, Z., Li, W., Hao, X., Liu, Y., Cao, S., Liu, W., Gibson, S., Zhang, K., Xu, G. and Xu, R.(2019). Circulating tumor DNA methylation profiles enable early diagnosis prognosis prediction and screening for colorectal cancer . Science Translational Medicine 12(524). DOI: 10.1126/scitranslmed.aax7533